Synnemata Production Using Silkworm Variety, Female Yangwonjam by *Isaria tenuipes*

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This study was conducted to confirm the possible use of female Yangwonjam as a host for synnemata production of *Isaria tenuipes* in eight local areas in Korea. Silkworm pupation rate, infection rate and synnemata characteristics of *I. tenuipes* were examined. Normal silkworms had a higher pupation rate than silkworms inoculated with *I. tenuipes*. The pupae survival percentage of normal silkworm in cocoons was 92.5~97.6%, whereas it ranged from 91.1~95.6% in silkworms sprayed with *I. tenuipes*. Female Yangwonjam showed the highest survival percentage at 97.6% among the silkworm varieties tested. *I. tenuipes* infection rate of larvae of 5th instar newly-exuviated silkworms was 89.2~90.7% in the spring rearing season and 98.2~99.3% in the autumn rearing season. Synnemata production of *I. tenuipes* was excellent in female Yangwonjam with an incidence rate of 98.0% followed by male Yangwonjam (94.1%) and Baegokjam (93.3%) in the spring rearing season. Synnemata living weight ranged from 1.44~0.94 g in the spring rearing season. The female Yangwonjam had the heaviest synnemata weight (1.44 g) in the spring rearing season. The synnemata of *I. tenuipes* produced on pupae were white or milky-white in color, and were similar in shape and color to wild synnemata collected in Korea.

KEYWORDS : *Bombyx mori*, *Isaria tenuipes*, Synnemata, Yangwonjam

Sericulture was one of the most important cottage industries in Korea during the 1960s and 1970s. Many researchers have tried to breed the varieties of silkworm (*Bombyx mori* L.) to improve the quantity and quality of raw silk. But, after the decline of the sericulture industry, attention shifted to health foods or dietary supplements such as silk powder and male pupa. These demands have forced the development of the new silkworm varieties suitable for special purposes. Recently, new silkworm varieties have been bred for these purposes in Korea. These varieties include Hachojam with non-cocooning silkworm variety for synnemata production of *Cordyceps* mushrooms [1], golden silk-producing yellow color cocoon [2], and Eolrukmal and Hukpyobeom with peculiar marks in the larval stage [3]. Also, Yangwonjam with both parent sex-limited larval marking variety has been bred for mass production of male pupae in Korea [4]. Male Yangwonjam pupae command a good market price due to their pharmaceutical value, whereas female pupae have no special use.

Seeking to augment the value of female pupae, the present study sought to use female pupae as a host to

produce fruiting bodies of *Isaria tenuipes* (formerly *Paecilomyces tenuipes*). *I. tenuipes* is a common entomogenous fungus found in many mountainous areas of Korea [5]. The fruiting body of this fungus is called snowflake Dongchunghacho due to its appearance. It has been reported to possess compounds of biological and pharmaceutical value, such as adenosine and N⁶-(2-hydroxyethyl) adenosine [6, 7]. Dongchunghacho mushrooms have been used as a traditional folk medicine or as a food ingredient to strengthen the immune system and regain energy, similar to the tonic functions of ginseng (*Panax* spp.) for hundreds of years in Far East Asian countries [8]. We investigated the pupation rate of female Yangwonjam, and the rates of infection and incidence of this fungus to achieve fruiting body formation. The results confirm the potential of female Yangwonjam as a host for the synnemata production of *I. tenuipes*.

Materials and Methods

Host insect. Yangwonjam is an F1 hybrid between the Japanese race Jam 143 and the Chinese race Jam 144 that

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Fig. 1. Larvae of 5th instar newly-exuviated silkworms. A, Female Yangwonjam larvae of 5th instar with crescent lunar marking; B, Male Yangwonjam larvae.

was bred in 1999 [4]. The marked larvae are females and plane larvae are males (Fig. 1A and 1B). The Japanese race pure line Jam 143 (breeding line JS 129) was crossed between stock lines of H5R1 and P8503 in 1993, and the Chinese pure line Jam 144 (breeding line CS98) was crossed between stock lines of M 8312 and 8190 in 1984. Baegokjam is a variety generated by a single cross F1 hybrid between the Japanese race Jam 123 and the Chinese race Jam 124 in 1982. The Japanese pure line Jam 123 was selected from stock lines of L 377 and 7603, and the Chinese race pure line Jam 124 was a hybrid between breeding lines of 7708 and 7268. The crossed silkworms were reared with natural mulberry leaves according to the silkworm rearing guide book of the National Academy of Agricultural Science (NAAS), Rural Development Administration (RDA), Korea.

Fungal strain. The *I. tenuipes* strain used in this study was isolated from conidiospores of synnemata collected at Mt. Songni, Chungcheongbukdo Province, Republic of Korea, on July 2, 2003. The specimen and strain were

preserved at the NAAS fungal herbarium.

Inoculum preparation. The conidiospores of *I. tenuipes* used for inoculation into silkworms were obtained from unconverted rice grain medium. This medium was made by mixing 150 g of unconverted rice grain, 15 g of silkworm pupae powder, and 80~100 mL of distilled water. The mixture was poured into a 500 mL flask. Each flask was autoclaved at 121°C for 20 min and inoculated with 5 mm mycelial discs of *I. tenuipes* from the growing margins on potato dextrose agar. The inoculated media were cultured at 25°C for 30 days and shaken at 3-day intervals to produce mass conidia [9]. The conidiospores formed on unconverted rice grain media were harvested using a sterile inoculation loop and added with 0.2 mL of 0.02% Tween-20 solution to uniformly disperse the spore suspension.

Inoculation. The concentration of the spore suspension was adjusted with sterilized distilled water to 10^8 spores/mL, and the number of spores was counted with a

Fig. 2. Spraying of inoculum onto silkworms and silkworm cocoons. A, Spraying inocula; B, Cocoons 11 days after mounting.

hematocytometer (Superior, Marienfeld, Germany). The spore suspension was added to starch syrup to promote easy attachment on the silkworm; the suspension was inoculated on each larva of 5th instar newly-exuviated silkworms using a previously-described spraying method [9]. Each test suspension was sprayed three times at 12-hr intervals (Fig. 2A). The sprayed silkworms were reared with natural mulberry leaves until mounting of the silkworm, as directed by the silkworm rearing guidebook of the NAAS.

Induction of endosclerotium and synnemata. The cocoons were harvested at 11 days after mounting from the cocoon frames (Fig. 2B). The silkworm pupae were picked out from the cocoons after cutting the tops with a knife the following day. The pupation rate was calculated with the number of healthy pupae within cocoons that were made after mounting of the mature larvae. The infected pupae were placed on a wet cotton cloth at 1 cm square distance in culture trays. Each tray with the infected pupae was kept in a growth room controlled at 20~22°C and a relative humidity of about 95% under a dark condition to induce synnemata formation. The containers were supplied with water periodically to prevent excessive drying.

Results and Discussion

Pupation rate. The pupae survival percentage of normal silkworm cocoons was 92.5~97.6% (Table 1). Healthy silkworm pupae (n = 1,430) were harvested from 1,465

silkworms in female Yangwonjam (97.6%), 1,361 silkworm pupae from 1,423 in male Yangwonjam (95.6%), and 1,356 silkworm pupae from 1,466 in Baegokjam (91.1%). There were significant differences in the pupation rate ($p < 0.05$) between Yangwonjam and Baegokjam in normal silkworms. The pupae survival percentage ranged from 91.1~95.6% in the silkworms sprayed with *I. tenuipes* (Table 2). Healthy silkworm pupae (n = 1,413) were harvested from 1,478 silkworms in female Yangwonjam (95.6%), 1,346 silkworm pupae from 1,472 in male Yangwonjam (91.4%), and 1,339 silkworm pupae from 1,470 in Baegokjam (91.1%). Female Yangwonjam had the highest survival percentage with 95.6% among the silkworm varieties tested. There were no significant differences in the pupation rate ($p < 0.05$) between male Yangwonjam and Baegokjam. Female Yangwonjam showed a higher survival percentage than the 66.9% value for Hachojam silkworm reported previously [10].

As is normal silkworms, the sprayed silkworms spun cocoons around themselves and become pupae within the cocoon (Fig. 2B). But, some of the silkworms died within the cocoon or missed from the cocoon frame during the rearing period for various reasons. Normal silkworms had a higher pupation rate than the silkworms inoculated with *I. tenuipes*, in agreement with a previous study that used a different silkworm variety [10].

Infection rate. Infection rate of *I. tenuipes* into larvae of 5th instar newly-exuviated silkworms was 89.2~90.7%

Table 1. Pupation rate of normal silkworm varieties

Silkworm variety	No. of silkworms tested	No. of silkworms pupated	Pupation rate (%)
Yangwonjam (female)	1,465	1,430	97.6 ^a
Yangwonjam (male)	1,423	1,361	95.6 ^a
Baegokjam	1,466	1,356	92.5 ^b

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Table 2. Pupation rate of silkworm varieties inoculated with *Isaria tenuipes*

Silkworm variety	No. of silkworms tested	No. of silkworms pupated	Pupation rate (%)
Yangwonjam (female)	1,478	1,413	95.6 ^a
Yangwonjam (male)	1,472	1,346	91.4 ^b
Baegokjam	1,470	1,339	91.1 ^b

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Table 3. Infection rate of *Isaria tenuipes* by silkworm variety in spring rearing season

Silkworm variety	No. of silkworms tested	No. of pupae infected	Infection rate (%)
Yangwonjam (female)	1,413	1,282	90.7 ^a
Yangwonjam (male)	1,346	1,200	89.2 ^a
Baegokjam	1,339	1,211	90.4 ^a

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Table 4. Infection rate of *Isaria tenuipes* by silkworm variety in autumn rearing season

Silkworm variety	No. of silkworms tested	No. of pupae infected	Infection rate (%)
Yangwonjam (female)	1,414	1,389	98.2 ^a
Yangwonjam (male)	1,370	1,360	99.3 ^a
Baegokjam	1,391	1,367	98.3 ^a

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Fig. 3. Silkworm pupae infected with *Isaria tenuipes*. A, Pupae mummified inside silkworm cocoon; B, Pupae filled with *Isaria* mycelium.

in the spring rearing season, and 98.2~99.3% in the autumn rearing season. No significant difference was observed in the infection rate among the silkworm varieties tested. In the spring rearing season, infection rates of *I. tenuipes* were 90.7% (n = 1,413) in female Yangwonjam, 89.2% (n = 1,346) in male Yangwonjam, and 90.4% (n = 1,339) in Baegokjam (Table 3). In the autumn rearing season, infection rates were 98.2% (n = 1,414) in female

Yangwonjam, 99.3% (n = 1,370) in male Yangwonjam, and 98.3% (n = 1,391) in Baegokjam (Table 4). Female Yangwonjam showed an equal infection rate with the Hachojam silkworm variety (99.8%) [10].

The spores of entomopathogenic fungi attach to the larval state of the insect and penetrate into the larval body. The mycelium develops inside the body of the insect by feeding on the internal nutrients until it completely occupies

Table 5. Synnema formation of *Isaria tenuipes* by silkworm variety in spring rearing season

Silkworm variety	No. of silkworms tested	No. of pupae produced synema	Incidence rate (%)
Yangwonjam (female)	1,282	1,257	98.0 ^a
Yangwonjam (male)	1,200	1,129	94.1 ^b
Baegokjam	1,211	1,130	93.3 ^b

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Table 6. Synnema formation of *Isaria tenuipes* by silkworm variety in autumn rearing season

Silkworm variety	No. of silkworms tested	No. of pupae produced synema	Incidence rate (%)
Yangwonjam (female)	1,389	1,389	100 ^a
Yangwonjam (male)	1,360	1,360	100 ^a
Baegokjam	1,367	1,360	99.5 ^a

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan's Multiple Range Test.

Fig. 4. Synnemata formation of *Isaria tenuipes* on the infected silkworm pupae. A, Mycelia induced on silkworm pupae; B, Synnemata formation on pupae with time.

the interior of the organism with its hyphae [11, 12]. Presently, pupae inoculated with *I. tenuipes* became hard and filled with the fungal mycelium internally, whereas the non-infected pupae were soft (Fig. 3).

Synnemata formation. Synnemata were induced from the infected pupae in the growth room with a temperature of 18~20°C and relative humidity of about 95% in the dark. Synnemata production of *I. tenuipes* was the best in female Yangwonjam with an incidence rate of 98.0% (n = 1,282), followed by male Yangwonjam with 94.1% (n = 1,200), and Baegokjam with 93.3% (n = 1,211) in the spring rearing season (Table 5). But, there was no significant difference in synnemata formation among the silkworm varieties in the autumn rearing season (Table 6). Primodia were induced out of pupae filled with *I. tenuipes* mycelium, and synnemata were longer and thicker if cultivating conditions were favorable (Fig. 4).

Synnemata characteristics. The synnemata living weight ranged from 1.44~0.94 g (Table 7). Female Yangwonjam had the heaviest synnema weight (1.44 g and 1.37 g), followed by Baegokjam (1.40 g and 1.22 g), and male Yangwonjam (0.94 g and 1.05 g) in the spring and autumn rearing season, respectively. There were significant

differences in the synnema living weight ($p < 0.05$) among the silkworm varieties in the autumn rearing season.

Synnemata produced in this experiment were white or milky-white in color (Fig. 5). Sung reported that this fungus collected in Korea produces conspicuous synnemata which are simple, furcate or irregularly branched, and usually up to 45 mm in length; the stipe is mostly yellow-brown to brown and terminates in a white to yellow fertile head [13]. Our results agree with these characteristics, indicating that synnemata produced in female Yangwonjam pupae were similar in shape and color to wild synnemata collected in Korea.

In this study, female Yangwonjam was selected as a new host of *I. tenuipes* for synnemata production following Hachojam reported by Kang *et al.* [10]. In particular, female Yangwonjam showed high pupation rate and heavier pupa weight, which should produce labor savings.

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Table 7. Characteristics of synnemata by silkworm variety

Silkworm variety	Synnemata living weight (g)	
	Spring rearing season	Autumn rearing season
Yangwonjam (female)	1.44 ^a	1.37 ^a
Yangwonjam (male)	0.94 ^b	1.05 ^c
Baegokjam	1.40 ^a	1.22 ^b

Means within columns followed by the same letter are not significantly different at the 0.05 level of probability determined by Duncan’s Multiple Range Test.

A

B

Fig. 5. Synnemata culture of *Isaria tenuipes* from infected silkworm pupae. A, Synnemata induction on culture tray; B, Synnemata formed on female Yangwonjam pupae.

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